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# Persistence of Biomarker ATP and ATP-Generating Capability in Bacterial Cells and Spores Contaminating Spacecraft Materials under Earth Conditions and in a Simulated Martian Environment▽

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**Most planetary protection research has concentrated on characterizing viable bioloads on spacecraft surfaces, developing techniques for bioload reduction prior to launch, and studying the effects of simulated martian environments on microbial survival. Little research has examined the persistence of biogenic signature molecules on spacecraft materials under simulated martian surface conditions. This study examined how endogenous adenosine-5′-triphosphate (ATP) would persist on aluminum coupons under simulated martian conditions of 7.1 mbar, full-spectrum simulated martian radiation calibrated to 4 W $m^{-2}$ of UV-C (200 to 280 nm), −10°C, and a Mars gas mix of $CO_2$ (95.54%), $N_2$ (2.7%), Ar (1.6%), $O_2$ (0.13%), and $H_2O$ (0.03%). Cell or spore viabilities of *Acinetobacter radioresistens*, *Bacillus pumilus*, and *B. subtilis* were measured in minutes to hours, while high levels of endogenous ATP were recovered after exposures of up to 21 days. The dominant factor responsible for temporal reductions in viability and loss of ATP was the simulated Mars surface radiation; low pressure, low temperature, and the Mars gas composition exhibited only slight effects. The normal burst of endogenous ATP detected during spore germination in *B. pumilus* and *B. subtilis* was reduced by 1 or 2 orders of magnitude following, respectively, 8- or 30-min exposures to simulated martian conditions. The results support the conclusion that endogenous ATP will persist for time periods that are likely to extend beyond the nominal lengths of most surface missions on Mars, and planetary protection protocols prior to launch may require additional rigor to further reduce the presence and abundance of biosignature molecules on spacecraft surfaces.**

The search for life on Mars has been identified as a National Aeronautics and Space Administration (NASA) Exploration Program goal of the highest importance (2). Exactly what to look for as evidence of life is currently unknown because no martian life has yet been detected, but careful consideration of the commonalities in all life from Earth permits some general assumptions about putative martian life (2). One such presumed characteristic is the use of organic molecules in biochemical processes, which may or may not have the same chemical structure as terrestrial biomolecules. Testing for biosignatures that are found ubiquitously in natural environments (nucleic acids, lipids, central metabolites, etc.), and which can be quantified with sensitive analytical methods amenable to automation, is a natural starting point into the search for martian life. If such searches result in the identification of molecules of clear biotic origin, a strong case for life detection would be made. For example, organic biomarkers, such as the amino acids glycine and D-alanine, have been proposed as candidate biomarkers and have been shown to exhibit long half-lives under simulated Mars surface conditions (6, 10, 33, 34).

The cellular metabolite adenosine-5′-triphosphate (ATP) exhibits a number of properties lending it importance as a top-priority (category A) biosignature in life detection experiments (20). First, all living organisms on Earth transform energy, regardless of the source, into chemical energy by synthesizing ATP. Second, ATP is ubiquitous in living cells, present in growing cells (16), and released from cells when they are killed by heat, disinfectants, or other treatments that disrupt the integrity of the cell envelope (36). Third, ATP has long been used as an extant biogenic marker, largely because a sensitive luciferin/luciferase assay has been widely available for more than 30 years (reviewed in reference 31) that is amenable to automation for use during robotic in situ or sample return missions. Fourth, measurements of ATP in low-bioload scenarios considered Mars analogs, such as Antarctic soils, have demonstrated that ATP could be used to detect the presence of sparse microbial populations in situ (3, 4).

In addition to its potential importance as a biosignature for Mars life detection missions, ATP has also been proposed to serve as a biomarker to measure spacecraft cleanliness for planetary protection purposes (36). This second use becomes particularly important if ATP is used as a biosignature for in situ Mars life detection experiments, to ensure that the hardware and reagents for sampling and assay are free from prior ATP contamination. Currently, the method used by NASA for measuring bacterial contamination relies on the enumeration of cultivable aerobic spore-forming bacteria (21). It is foreseeable that criteria for the cleanliness of orbiters and rovers might become more stringent and be expanded to set limits not only for bioloads but for detectable biosignatures in order to

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avoid obscuring positive biosignature findings on solar system bodies.

It has been supposed that one drawback of ATP as a biosignature molecule is that it does not persist in the environment outside cells due to the intrinsic lability of the high-energy γ-phosphate group (20). However, evidence from recent experiments in our lab contradicts this supposition. We observed that purified ATP applied to spacecraft-qualified materials and exposed to simulations of the Mars environment was surprisingly stable, persisting with a half-life of 22 martian sols at −10°C even when exposed to full-spectrum UV-visible-infrared (UV-VIS-IR) radiation characteristic of the martian surface (23). In addition, a Mars surface irradiance model (15) was used to estimate residence times for ATP on the upper and lower surfaces of rovers and landers at any martian latitude. The model predicted that pure ATP could persist on Mars landers and rovers for extended periods, up to decades (23).

A number of microorganisms have been identified as frequent contaminants of spacecraft and their assembly facilities, and several of the microbial isolates exhibit elevated levels of resistance to common spacecraft disinfectants such as UV, gamma radiation, and hydrogen peroxide vapor (8, 11, 12, 35). Such microorganisms are more likely to contaminate Mars-bound spacecraft and survive the Earth-to-Mars transit (reviewed in reference 5). Given these considerations, it is important to ascertain the permanence of detectable ATP under martian conditions. Previous work (23) has demonstrated that exogenous ATP (i.e., ATP present as extracellular biogenic molecules) could persist for long periods of time under simulated Mars surface conditions. The primary objective of the current research was to extend this research to investigate the stability of endogenous ATP present within intact cells of common spacecraft contaminants located on the surface of spacecraft materials and exposed to simulated martian conditions. In addition, a significant proportion of microbial contaminants found on spacecraft are highly resistant endospores of the species *Bacillus* (9, 11, 12), and bacterial endospores are preprogrammed to produce a burst of ATP when germination is triggered. Therefore, a secondary objective of the research was to determine whether this burst of ATP would occur in endospores even after they endured lethal exposures to simulated Mars surface conditions, including full-spectrum solar radiation.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *Escherichia coli* K-12 and *Bacillus subtilis* 168 are strains WN120 and WN131, respectively, from our laboratory strain collection. *Acinetobacter radioresistens* strain 50v1 (35) and *Bacillus pumilus* strain SAFR-032 (12, 35) were both generously donated by K. Venkateswaran, NASA Jet Propulsion Laboratory. *Pseudomonas fluorescens* was obtained from M. Roberts, Dynamac Corp. For routine growth of cells, Luria-Bertani (LB) medium (14) was used. Spores were produced in Schaeffer sporulation medium (22). Liquid medium was solidified for plates with 1.5% agar. *B. subtilis* and *E. coli* cells were cultivated at 37°C; *A. radioresistens* and *B. pumilus* were cultivated at 30°C, and *P. fluorescens* was cultivated at 25°C. For exposure experiments, vegetative cells were grown in liquid LB medium overnight at the appropriate growth temperature, harvested by centrifugation in a microfuge (10,000 × *g*, 2 min), resuspended in sterile distilled water, and diluted to an appropriate, predetermined cell density. Spores of *B. subtilis* and *B. pumilus* were produced by incubation for at least 4 days on solid Schaeffer sporulation medium, harvested, and purified by lysozyme and buffer washing as described previously (18). Spores were heat shocked (80°C, 10 min), titrated by serial tenfold dilutions and plate counts on solid LB medium, and stored in distilled water at 4°C. It was determined by phase-contrast microscopy that spore preparations consisted of >99% phase-bright spores. Spores were germinated in liquid 1× Spizizen salts (30) supplemented with the germinant L-alanine (10 mM, final concentration) and tryptophan (50 μg/ml, final concentration). For particular germination experiments, sodium fluoride (NaF) or chloramphenicol (Cm) were added to final concentrations of 10 mM or 25 μg/ml, respectively.

**Sample preparation.** Spacecraft-qualified aluminum-6061 coupons (10 mm by 20 mm by 1 mm) were treated with a chromium-oxide coating called Chemfilm (23, 26) to reduce oxidation of aluminum surfaces. Coupons were heat sterilized at 130°C for 24 h and cooled to room temperature. Aliquots containing ~$2 \times 10^7$ cells or spores in 50 μl were spotted evenly onto coupons and air dried at 37°C for 1 h before exposure to test conditions.

**Mars environmental simulation and sample exposure conditions.** Samples were exposed to conditions simulating Mars atmospheric composition, pressure, solar radiation, and temperatures in a Mars simulation chamber (MSC). The MSC system has been previously described in detail (23). Coupons containing vegetative cells or spores secured in sample holders were placed inside the MSC. The Mars chamber was then sealed, the pressure was brought down to 1 mbar, and a gas mixture of $CO_2$ (95.54%), $N_2$ (2.7%), Ar (1.6%), $O_2$ (0.13%), and $H_2O$ (0.03%) was introduced to a pressure of 7 ± 0.1 mbar; this gas composition and pressure closely matches the martian atmosphere (19). After 10 min, the temperature was set at −10°C ± 1.5°C, and the samples were allowed to stabilize for an additional 20 min. To simulate Mars solar radiation exposures, samples were irradiated with a fluence rate of UV-VIS-IR light (200 to 2,500 nm) calibrated to 4.1 W/m$^2$ UV-C (200 to 280 nm), delivered from a 1,000-W xenon-arc lamp as described previously (23, 27). Simultaneously exposed controls consisted of one parallel set of samples exposed to the same simulated Mars conditions except shielded from simulated solar radiation by wrapping sample holders in aluminum foil, and a second parallel set exposed to Earth conditions wrapped with aluminum foil for light protection and placed on the benchtop at ~23°C. After exposure, each coupon was placed into a 13-mm-diameter glass test tube containing 2 ml of phosphate-buffered saline (PBS; 10 mM potassium phosphate, 150 mM NaCl [pH 7.4]) (18). Cells or spores were removed from coupons by vortexing vigorously for 5 s, resting for 5 min, and vortexing again for 5 s.

**Viability assays.** Aliquots of cells or spores were removed from vortex-mixed sample tubes, diluted serially 10-fold in PBS, and plated on LB medium. Colonies were counted after 24 to 48 h of incubation at the appropriate temperature, and the survival was calculated.

**ATP assays.** Portions (50 μl) were removed from vortex-mixed suspensions and assayed for ATP by luciferin/luciferase assay using a BacTiter-Glo microbial cell viability assay kit (Promega, Madison, WI) as described previously (23). The luminescence signal was measured in a luminometer (Harta Instruments, Gaithersburg, MD), using 96-well microtiter plate format, glow detection mode, and a dwell time of 1 s. In all experiments, a well containing medium without microorganisms was included to obtain a value for background luminescence. Luminescence signals are expressed in relative light units (RLU).

**ATP production during spore germination.** In order to calibrate the system, initial experiments were performed to measure ATP generation during germination of *B. pumilus* or *B. subtilis* spores. Suspensions of $2 \times 10^7$ spores of each strain were added to 16-mm-diameter glass test tubes containing 2 ml of 1× Spizizen salts (30) with tryptophan and the potent spore germinant L-alanine. Tubes were vortex mixed for 5 s, placed in a water bath on an angle for aeration and shaken vigorously (300 rpm) at 37°C to initiate germination. Aliquots (50 μl) were removed at 5-min intervals for ATP measurements. Inhibition of ATP production during spore germination was measured as described above, but in the presence of either Cm or NaF, added to final concentrations of 25 μg/ml and 10 mM, respectively.

**ATP in spores germinated after exposure to simulated Mars conditions.** After exposure to experimental conditions, coupons containing spores were placed directly into 16-mm-diameter glass test tubes containing 2 ml of LB medium containing 10 mM L-alanine. Tubes were vortex mixed for 5 s, placed in a water bath on an angle for aeration, and shaken vigorously (300 rpm) at 37°C to initiate germination. Samples (50 μl) were removed from the germinating spore suspensions at various times for ATP assay, as described above.

**Statistical analysis of data.** Data points consist of triplicate determinations performed in duplicate experiments. Basic statistical parameters and analyses of variance (ANOVA) were computed by using commercial statistics software (Kaleidagraph, version 3.6.2; Synergy Software, Reading, PA). Differences with $P$ values of $\leq 0.05$ were considered statistically significant.

TABLE 1. Inactivation of cells and spores by air drying on chemfilm-treated Al 6061 coupons

| Species | Cells or spores | No. of cells or spores[a] per coupon: | | Survival ($N/N_0$) |
|---|---|---|---|---|
| | | Before air drying ($N_0$) | After air drying ($N$) | |
| *E. coli* K-12 | Cells | $6.48 \times 10^7$ | $4.00 \times 10^6$ | 0.06 |
| *P. fluorescens* pTOL | Cells | $3.08 \times 10^7$ | $<4 \times 10^{1b}$ | $<1.3 \times 10^{-6}$ |
| *A. radioresistens* 50v1 | Cells | $3.16 \times 10^8$ | $1.47 \times 10^7$ | 0.04 |
| *B. subtilis* 168 | Cells | $1.24 \times 10^7$ | $3.20 \times 10^2$ | $2.58 \times 10^{-5}$ |
| | Spores | $2.00 \times 10^7$ | $2.05 \times 10^7$ | 1.03 |
| *B. pumilus* SAFR-032 | Cells | $5.00 \times 10^7$ | $4.10 \times 10^4$ | $8.2 \times 10^{-4}$ |
| | Spores | $2.00 \times 10^7$ | $2.26 \times 10^7$ | 1.13 |

[a] Average of two determinations.
[b] Lower limit of detection of viability assay: 40 CFU/ml.

TABLE 2. ATP content in cells and spores before and after air drying on chemfilm-treated Al 6061 coupons

| Species | Cells or spores | RLU per coupon[a]: | | ATP persistence after drying ($ATP/ATP_0$) |
|---|---|---|---|---|
| | | Before air drying ($ATP_0$) | After air drying (ATP) | |
| *E. coli* K-12 | Cells | $2.30 \times 10^7$ | $3.00 \times 10^6$ | 0.13 |
| *P. fluorescens* pTOL | Cells | $1.54 \times 10^7$ | $1.82 \times 10^6$ | 0.12 |
| *A. radioresistens* 50v1 | Cells | $2.17 \times 10^7$ | $1.15 \times 10^7$ | 0.53 |
| *B. subtilis* 168 | Cells | $1.50 \times 10^7$ | $3.93 \times 10^5$ | 0.11 |
| | Spores | $8.02 \times 10^4$ | $4.49 \times 10^4$ | 0.56 |
| *B. pumilus* SAFR-032 | Cells | $8.06 \times 10^5$ | $4.10 \times 10^5$ | 0.51 |
| | Spores | $1.08 \times 10^5$ | $4.01 \times 10^4$ | 0.37 |

[a] Average of at least two determinations.

## RESULTS AND DISCUSSION

**Calibration of the ATP assay.** In order to correlate the number of viable cells with the amount of ATP present, the five bacterial strains were grown separately overnight in liquid LB medium. Each culture was diluted serially 10-fold in PBS from $10^0$ to $10^{-7}$. Viable counts and ATP content were determined from the same sample of each dilution. In all cases, a linear correlation over 4 orders of magnitude between the luminescence signal and the number of viable cells was observed (data not shown). The lower limits of detection were approximately 44, 30, 19, 2, and 28 vegetative cells for *E. coli*, *B. subtilis*, *B. pumilus*, *A. radioresistens*, and *P. fluorescens*, respectively. In contrast, the content of ATP in dormant bacterial endospores was determined to be very low, a finding consistent with the data of Setlow and Kornberg (29). In order to obtain luminescence above background levels, it was necessary to assay $2 \times 10^7$ *B. subtilis* spores or $1.28 \times 10^7$ *B. pumilus* spores.

**Viability and ATP levels of cells and spores deposited on coupons.** Before assessing the survival of the bacterial species to simulated Martian conditions, we determined their survival to simple air drying on the surface of aluminum coupons. The results (Table 1) indicated that vegetative cells of all tested species suffered a loss in viability after drying on the surface of aluminum coupons. For *P. fluorescens*, the drop in viability by air drying was so severe, more than 6 orders of magnitude, that this organism was eliminated from further testing. Not surprisingly, bacterial endospores did not lose any viability as a result of short-term drying on coupons (Table 1). Microscopic direct counts of bacterial suspensions recovered from the coupons showed that for all microorganisms tested, the observed decreases in viability were not due to diminished cell recovery (data not shown).

In addition to determining the survival of cells from air drying on coupons, the ability to recover detectable ATP from samples after air drying was investigated. After cells or spores were air dried on aluminum coupons, the levels of detectable ATP were found to range between 10 and 50% of the amount of ATP measured in the cells or spores before air drying (Table 2). Thus, vegetative cells of different bacterial species showed widely varying, often drastic, reductions in viability upon air drying; in contrast, their constituent ATP was relatively stable to air drying.

**Inactivation of viability and ATP in vegetative cells and spores.** In order to determine how rapidly viability and ATP biosignature levels of spacecraft-contaminating microorganisms are inactivated on spacecraft surfaces under simulated Mars surface conditions, coupons were coated with suspensions of the test strains and exposed in parallel to Earth conditions or in the MSC in the presence or absence of simulated Mars UV, and their survival and ATP levels were determined. The species selected for testing were *A. radioresistens* 50v1 and *B. pumilus* SAFR-032, which were originally isolated as highly resistant contaminants of the Mars Odyssey spacecraft and the JPL Spacecraft Assembly Facility, respectively (9, 11, 12, 35).

Cells of *A. radioresistens* 50v1 were dried onto coupons and exposed for 7 days to Earth conditions or to simulated Mars conditions in the MSC in the absence or presence of continuous simulated Mars solar irradiation. For comparison purposes, initial ATP levels and viable counts of the original bacterial suspension were determined immediately after air drying on coupons. Simply exposing coupons to laboratory benchtop (i.e., Earth) conditions for 7 days did not affect the levels of ATP detected, although cell viability was decreased by an order of magnitude (Fig. 1A). After 7 days under Mars conditions without UV irradiation the levels of ATP detected were slightly but significantly higher than those in preexposed samples, even though no significant change in viability was observed (Fig. 1A). When the *A. radioresistens* samples were irradiated for 7 days under Mars conditions including full-spectrum simulated Mars UV-VIS-IR radiation, ATP levels were not significantly altered, despite a decrease in viability greater than 4 orders of magnitude (i.e., below the 40 CFU/ml lower threshold of sensitivity of the viability assay) (Fig. 1A).

Vegetative cells (Fig. 1B) and spores (Fig. 1C) of *B. pumilus* SAFR-32 were subjected to the same experimental conditions as described above for *A. radioresistens* 50v1. As seen with *A. radioresistens* 50v1, the ATP levels and viability of *B. pumilus* SAFR-032 cells or spores were relatively unaffected in all samples exposed for 7 days to Earth or simulated Mars conditions in the absence of simulated Mars solar radiation (Fig. 1B and C). However, although ATP levels remained relatively unchanged, the viability of *B. pumilus* SAFR-032 cells or spores

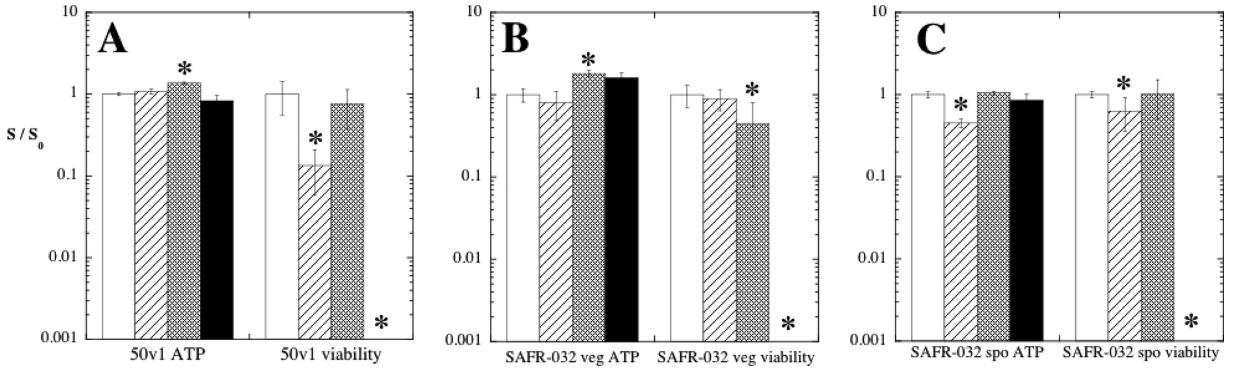


FIG. 1. Levels of ATP and viability in cells of *A. radioresistens* 50v1 (A) and *B. pumilus* SAFR-032 vegetative cells (B) and spores (C) on chemfilm-treated Al 6061 coupons. Samples were not exposed (□) or exposed for 1 week to: (i) Earth atmosphere, +23°C (▨); (ii) MSC, Mars atmosphere, −10°C, shielded from simulated Mars solar radiation (▩); (iii) MSC, Mars atmosphere, −10°C, exposed to full-spectrum simulated Mars solar radiation (■). The data are averages ± the standard deviations ($n = 6$). Asterisks denote significant differences from preexposed samples (ANOVA, $P < 0.05$). Note that the solid bars are absent in viability samples because no CFU were recovered from these samples (limit of sensitivity of $10^{-4}$ survival for panels A and B; $10^{-7}$ survival for panel C). The preexposure levels of ATP in *A. radioresistens* and in *B. pumilus* vegetative cells and spores were $6.89 \times 10^5$, $3.93 \times 10^5$, and $3.97 \times 10^4$ RLU, respectively.

was dramatically reduced below the detection limit of the assay (Fig. 1B and C). The results showed that ATP levels from vegetative cells or spores did not decrease after exposure to simulated Mars environmental regime equivalent to 7 days of continuous UV irradiation under Mars-normal conditions of pressure, temperature, and atmospheric composition. Using the Mars UV transfer models of Moores et al. (15), seven Earth days of continuous UV-C irradiation in the MSC system at 4.1 W $m^{-2}$ is the approximate equivalent of 40 sols of martian surface solar irradiation (23).

In order to extend these observations to longer exposure periods, triplicate sets of coupons containing *A. radioresistens* 50v1 cells were prepared; placed in the MSC either exposed to or shielded from simulated Mars solar radiation; and exposed for 7, 14, and 21 days with parallel Earth controls. Cell viability assays showed that after the first 7 days under Earth conditions, *A. radioresistens* viability decreased by 3 orders of magnitude and then remained relatively unchanged for up to 21 days (Fig. 2A). Cells placed in the MSC but shielded from simulated Mars solar irradiation experienced a steady drop in viability throughout the exposure period, losing only 2 orders of magnitude of viability by 21 days (Fig. 2A). Upon exposure in the MSC to the full suite of simulated Mars conditions, including simulated solar radiation, *A. radioresistens* 50v1 viability was completely abolished below the level of detection of the assay (40 CFU) within the first 7 days of exposure (Fig. 2A). Differences in cell viability at 21 days under all three treatments were highly significant as determined by ANOVA ($P < 0.01$). The survival rates of *A. radioresistens* at 7, 14, and 21 days under Mars pressure and temperature were greater than the Earth controls (Fig. 2A), a result that supports the conclusion that the lower temperature in the Mars controls maintained at −10°C under anoxic conditions inhibited the decline of viability noted in the Earth controls maintained at approximately 23°C.

Parallel measurement of ATP levels revealed that despite the large and variable decreases in viability of *A. radioresistens* 50v1 cells under the three environmental conditions tested, ATP levels after 21 days of exposure dropped by less than 1 order of magnitude: only 38, 14, and 65% in cells exposed to Earth, MSC shielded, and MSC exposed to simulated Mars solar radiation, respectively (Fig. 2B). It should be noted that the solar UV-C radiation dose after 21 Earth days in the MSC is equivalent to ~115 martian sols (23). When ATP started to

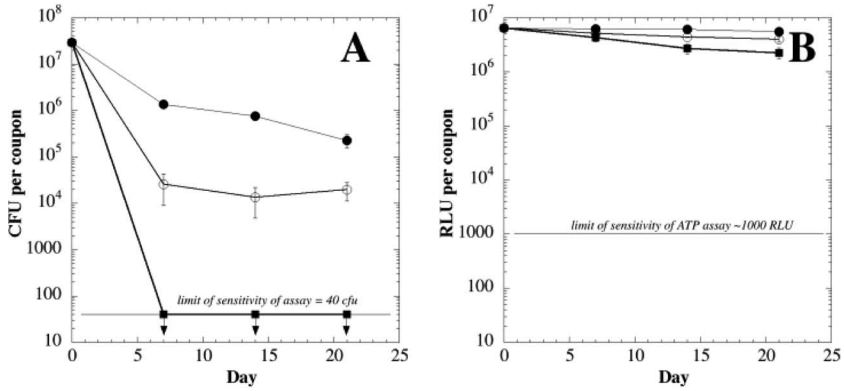


FIG. 2. Viability (A) and levels of ATP (B) of *A. radioresistens* 50v1 cells on coupons during 21 days of incubation at Earth conditions (○) and in the MSC shielded from (●) or exposed to (■) simulated Mars solar radiation. The data are averages ± the standard deviations ($n = 6$).

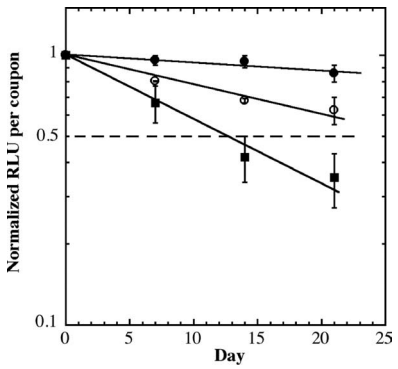


FIG. 3. Determination of endogenous ATP half-life (denoted by dashed line) in cells of *A. radioresistens* 50v1 under Earth conditions (○), in the MSC shielded from radiation (●), and in the MSC exposed to simulated Mars solar radiation (■). The data are averages ± the standard deviations ($n = 6$). The equations and correlation coefficients for the best-fit exponential curves of the data were (i) Earth, $y = 0.96677 \times e^{-0.022435x}$, and $R^2 = 0.96551$; (ii) MSC minus irradiation, $y = 1.0086 \times e^{-0.0067284x}$, and $R^2 = 0.87917$; and (iii) MSC plus irradiation, $y = 0.95949 \times e^{-0.051347x}$, and $R^2 = 0.98519$.

be tested as an indicator of biological contamination in assessing spacecraft cleanliness for planetary protection purposes, it was found that a quantitative calibration between the amount of ATP and the total cell number in a sample was not possible, because the amount of ATP per cell varies greatly depending on the cell type and physiological state (36). Our data demonstrate, furthermore, that over extended periods of time on the martian surface, nonviable spacecraft contaminants will likely retain much of their endogenous ATP biosignature on solar UV-exposed surfaces.

How long can endogenous ATP persist under Mars surface conditions? In a previous study (23), we exposed purified ATP on spacecraft surfaces in the MSC to temperatures spanning the minimum (−80°C) and high (−10°C) average summertime temperatures recorded by the Viking landers, and the maximum Mars surface temperature in southern equatorial regions (20°C) (19), in order to calculate ATP longevity at virtually any location on the surface of Mars. In a similar manner, the endogenous ATP degradation data obtained in the MSC at −10°C (Fig. 2B) were normalized and plotted versus time. It was observed that endogenous ATP degradation over time followed kinetics that conformed to exponential best-fit curves with high correlation coefficients (Fig. 3). From the best-fit equations it was determined that the half-lives of endogenous ATP in cells of *A. radioresistens* 50v1 were 29.4 days under Earth conditions and 104.3 days in the MSC when protected from simulated Mars solar UV irradiation (Fig. 3; note that these values are extrapolations using the best-fit equations). When exposed to simulated Mars solar radiation in the MSC, ATP in cells exhibited a half-life of 12.7 days, which is the equivalent of being exposed to surface UV-C radiation for ~70 martian sols (15). Thus, the half-life of endogenous ATP in cells is about 3.2 times the half-life calculated for purified ATP exposed to the full suite of conditions in the MSC including simulated Mars surface radiation (23).

**ATP generation in germinating spores.** Measured levels of ATP were at least an order of magnitude higher in vegetative cells than in dormant spores (Fig. 1 legend). This is consistent with previous data showing that dormant spores contain low endogenous levels of ATP (28, 29, 36). Although dormant bacterial spores contain little endogenous ATP, they do contain large amounts of endogenous ADP and AMP; furthermore, abundant high-energy phosphate is stored in the dormant spore as a large depot of 3-phosphoglyceric acid. In the first minutes of spore germination, the phosphate stored in 3-phosphoglyceric acid is rapidly transferred to ATP by the lower branch of glycolysis, mediated by the enzymes phosphoglycerate mutase, enolase, and pyruvate kinase. These enzymes are prepackaged in the dormant spore; hence, ATP generation during germination does not require de novo protein synthesis (29). Therefore, if terrestrial spores contaminating spacecraft were rendered nonviable by exposure to Mars solar UV radiation but were still induced to initiate germination (by nutrient and water addition, for example), then the resulting burst of ATP produced could generate a false-positive signal in an ATP-based life detection experiment. With this possibility in mind, we tested the ability of spores exposed to simulated Mars conditions to produce ATP upon subsequent triggering of germination.

First, it was necessary to determine whether ATP generation during germination of *B. pumilus* SAFR-032 spores was similar to ATP generation in the well-characterized *B. subtilis* benchmark laboratory organism. Spores ($2 \times 10^7$) were placed in germination medium in triplicate, and the kinetics of ATP generation were monitored for the first 20 min of germination. In spores of *B. subtilis* strain 168, ATP was generated rapidly during the first 5 to 10 min of germination (Fig. 4A). The addition of the protein synthesis inhibitor Cm did not inhibit ATP generation (Fig. 4A), as would be expected because the enzymes of the lower branch of glycolysis are prepackaged in dormant spores (29). However, the addition of NaF, a potent inhibitor of enolase (29), dramatically inhibited ATP production in germinating spores by ~75% (Fig. 4A). The kinetics of ATP production and its response to Cm and NaF addition in germinating spores of *B. pumilus* SAFR-032 were experimentally confirmed to be essentially identical to those of *B. subtilis* 168 (data not shown). Control experiments confirmed that neither NaF nor Cm were direct inhibitors of luciferase activity in the ATP assay itself (data not shown).

The total cumulative RLU generated during the course of spore germination reflects the total amount of ATP produced by a germinating spore population, and this value is more amenable to statistical analysis than the kinetics data (32). Equal numbers of spores ($2 \times 10^7$) of both *B. subtilis* 168 and *B. pumilus* SAFR-032 were germinated in triplicate, and their total ATP production over 20 min of germination was determined and compared by ANOVA (Fig. 4B). Germinating spore populations of both species behaved similarly in that Cm did not significantly affect ATP production, but NaF did significantly inhibit ATP production (Fig. 4B). However, ATP production in germinating *B. pumilus* SAFR-032 spores appeared to be somewhat more resistant to NaF inhibition (54% ± 5% inhibition) than in germinating *B. subtilis* 168 spores (75% ± 5% inhibition) (Fig. 4B). Also, it was surprising to observe that *B. pumilus* SAFR-032 spores produced nearly 7 times as much ATP as did the equivalent number of *B. subtilis* spores (Fig. 4B). The results of this series of experiments strongly indicate that ATP is

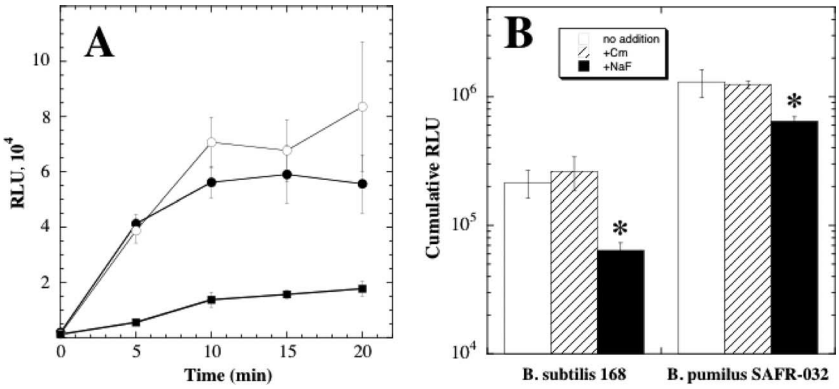


FIG. 4. (A) Kinetics of ATP production by germinating spores of *B. subtilis* 168 in SMM plus 10 mM L-alanine with no additions (●), Cm (25 μg/ml) (○), or NaF (10 mM) (■). (B) Cumulative ATP generated during the first 20 min of germination of spores of *B. subtilis* 168 (left) and *B. pumilus* SAFR-032 (right) in SMM plus 10 mM L-alanine with no additions (□), Cm (25 μg/ml) (▨), or NaF (10 mM) (■). The data are expressed as averages ± the standard deviations ($n = 6$). Asterisks denote significant differences by ANOVA ($P < 0.05$).

generated in germinating *B. pumilus* SAFR-032 spores by the same pathway as previously described for spores of *B. megaterium* (29) and *B. subtilis* (28).

**Inhibition of germination-induced ATP production in the MSC.** To determine the effects of simulated Mars conditions on the ability of spores to produce ATP during germination, triplicate samples of *B. pumilus* SAFR-032 spores were exposed for 1 week on coupons to Earth conditions and in the MSC in the absence or presence of simulated Mars solar irradiation. After exposure, the coupons were placed directly into germination medium, and the production of germination-induced ATP was measured for 120 min (Fig. 5). It was apparent that *B. pumilus* SAFR-032 spores exposed for 1 week in the MSC, but shielded from simulated Mars solar radiation, were still able to generate ATP during germination with kinetics indistinguishable from Earth-exposed controls (Fig. 5). In contrast, exposure of spores in the MSC to simulated Mars solar radiation for 1 week (equivalent to ~40 martian sols) completely abolished their ability to produce ATP upon subsequent germination (Fig. 5).

To investigate whether shorter exposures to simulated Mars solar radiation in the MSC would still allow for germination-triggered ATP production, triplicate samples of *B. subtilis* 168 and *B. pumilus* SAFR-03 spores were exposed to the Earth and simulated Mars environmental conditions, with or without simulated Mars solar radiation, for 8, 24, or 72 h (the equivalent of ~1.8, 5.5, and 16.5 martian sols of solar irradiation, respectively). Spores were then germinated for 120 min, their germination-induced ATP was measured, and cumulative ATP levels were calculated for each sample (Fig. 6). Again, exposure of *B. subtilis* 168 spores (Fig. 6A) or *B. pumilus* SAFR-03 spores (Fig. 6B) in the MSC, but shielded from simulated Mars solar irradiation, resulted in germination-induced ATP levels not statistically different from the parallel Earth-exposed control coupons. However, exposure in the MSC to simulated Mars solar radiation for as little as 8 h abolished the spores' ability to produce ATP during germination (Fig. 6).

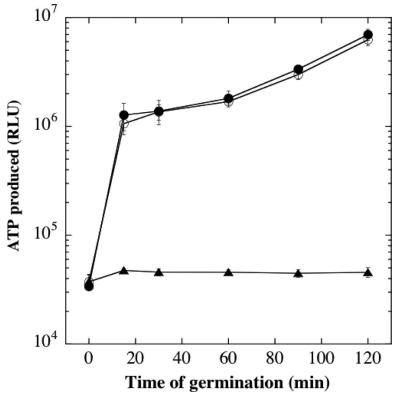


FIG. 5. Kinetics of ATP production during germination of *B. pumilus* SAFR-032 spores after incubation for 1 week under Earth conditions (●), in the MSC but shielded from simulated Mars solar radiation (○), or in the MSC exposed to simulated Mars solar radiation (▲). The data are expressed as averages ± the standard deviations ($n = 6$).

**Kinetics of inactivation of spore viability versus germination ATP production by simulated Mars solar radiation.** It has been established that simulated Mars solar radiation, particularly the UV component, is rapidly lethal to unshielded spores (24, 25), and further that some early spore germination events, such as the establishment of proton motive force and oxidation-reduction potential, were inactivated at a rate significantly slower than spore viability (32). From the above sets of experiments it was clear that exposure of spores to as little as 8 h of simulated Mars solar radiation was sufficient to completely abolish germination-induced ATP production (Fig. 6). Therefore, a time course experiment using much shorter UV exposure times was performed in order to measure the relative inactivation rates of spore viability and ATP production during germination.

In three independent determinations, *B. subtilis* 168 and *B. pumilus* SAFR-032 spores were exposed in liquid to simulated Mars solar radiation at the flux expected on the surface. Samples were removed at 0, 1, 2, 4, 8, 16, and 32 min and assayed for viability and germination-induced ATP production. In both species, spore viability was inactivated much more quickly than was the ability of spores to produce ATP upon triggering of spore germination (Fig. 7A). The lethal doses of simulated Mars solar irradiation required to inactivate 90% of spore viability or ATP-generating capability ($LD_{90}$) were computed.

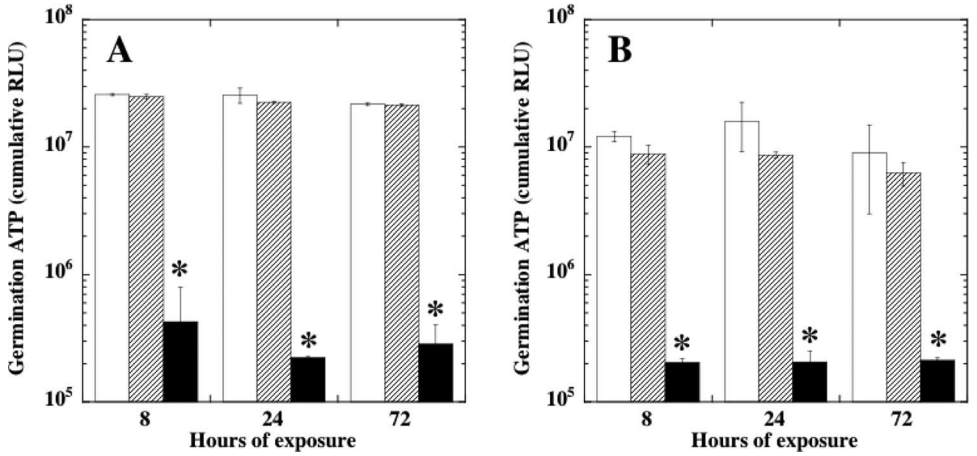


FIG. 6. Cumulative ATP produced during germination of *B. subtilis* 168 spores (A) and *B. pumilus* SAFR-032 spores (B). Samples were exposed for the indicated times to the following conditions: Earth atmosphere, 23°C (□); MSC, Mars atmosphere, −10°C, shielded from UV (▨); or MSC, Mars atmosphere, −10°C, full-spectrum Mars UV (■). The data are averages ± the standard deviations ($n = 6$). Asterisks denote significant differences as determined by ANOVA ($P < 0.05$).

The $LD_{90}$ values for inactivation of *B. subtilis* 168 and *B. pumilus* SAFR-032 spores were 0.283 ± 0.0167 and 1.633 ± 0.132 min, respectively; the differences in these values were highly significant as determined by ANOVA ($P < 0.0001$) (Fig. 7B). *B. pumilus* spores were 5.6-fold more resistant to simulated Mars solar irradiation than that of *B. subtilis* 168 spores, a finding in agreement with previous experiments using monochromatic 254-nm UV (12). In contrast, germination-induced ATP-generating ability under the same exposure conditions was found to exhibit $LD_{90}$s of 10.105 ± 0.618 and 10.109 ± 3.313 min for *B. subtilis* 168 and *B. pumilus* SAFR-032 spores, respectively, and these values were not significantly different by ANOVA ($P = 0.9988$ (Fig. 7B). Thus, inactivation of germination ATP production by *B. subtilis* 168 and *B. pumilus* SAFR-032 by 90% during simulated Mars solar irradiation required the equivalent of 35.7 and 6.2 times the $LD_{90}$ dose for viability, respectively. These data are in good agreement with previous results indicating that inactivation of the proton motive force and redox potential during spore germination also required severalfold-greater doses of simulated Mars solar radiation than did spore killing (32). The data from Fig. 7 indicate that the ability of a spore population to produce ATP during germination was inactivated by Mars solar irradiation at a rate of approximately 1 log per 10 min. It would therefore be expected that the ability of spores to generate ATP might be completely abolished (>6-log inactivation) by the first hour of solar exposure on the martian surface. In stark contrast, calculations from the data presented in Fig. 3 indicate that to

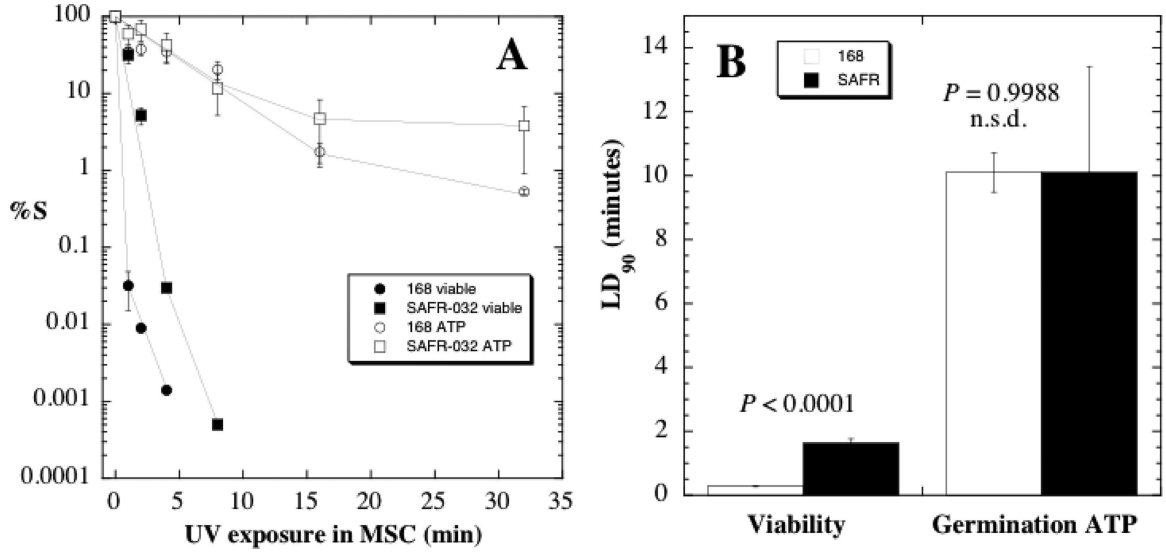


FIG. 7. (A) Inactivation kinetics of spore viability (solid symbols) and germination-induced ATP production (open symbols) of *B. subtilis* 168 spores (circles) and *B. pumilus* SAFR-032 spores (squares). Samples were exposed for the indicated times in liquid suspension to full-spectrum simulated Mars solar radiation. (B) Comparison of $LD_{90}$ values for viability (left) and germination ATP production (right) of *B. subtilis* 168 spores (□) and *B. pumilus* SAFR-032 spores (■). The data are averages ± the standard deviations ($n = 6$). n.s.d., no significant difference.

reduce preformed ATP inside cells or spores by >6 logs might require tens to thousands of sols on sun-exposed surfaces.

The search for extraterrestrial life inevitably prompts attempts to define very fundamental and elusive concepts such as the universal characteristics of life, and the nature of specific detectable components or properties that are uniquely biotic (see for example, references 13 and 20). For a variety of reasons enumerated in the introduction, ATP has been considered a good candidate proxy for the presence of extant or extinct life. However, forward contamination risks to missions destined to the martian surface extend beyond the potential survival of the contaminant cells themselves. First, the presence of viable cells or spores could negatively impact the success of metabolically based life detection systems. Second, even if cells and spores are inactivated by any one, or several, of the more than 12 biocidal factors on the surface of Mars (25), the biofilms and biogenic signature molecules left after cell death (such as ATP) may still confound life detection payloads. The present study examined the degradation of endogenous ATP present in, and protected by, cells or spores of three common spacecraft-contaminating microorganisms under simulated martian conditions. This work was an extension of a previous study (23) that examined the persistence of exogenous ATP on spacecraft surfaces under identically simulated Mars surface conditions. The results from both studies support the conclusions that (i) viable spacecraft contaminants will be rapidly inactivated by solar UV irradiation on Mars, but (ii) biogenic signature molecules may persist for considerably longer periods of time. These results are consistent with a wide range of literature on the survival of terrestrial microorganisms under simulated martian conditions (see references 5, 7, 8, 12, 17, 24, 25, and 32 and references therein). In addition, a few prior studies have demonstrated that biogenic signature molecules persist longer under Mars UV irradiation and atmospheric conditions than the viability of the microbial species producing the biosignature molecules (1, 15, 32). Thus, the surface of Mars should be considered extremely harsh for the survival of viable terrestrial microorganisms but may be considerably more benign to the persistence of biogenic signature molecules.

In earlier work (23), we predicted that exogenous ATP could persist on sun-exposed surfaces between 158 to 975 sols for equatorial and polar landing sites on Mars, respectively; and up to $3.2 \times 10^4$ sols for the undersides of landers or rovers. The present study extends persistence of biogenic molecules by a factor of 3.2 for endogenous ATP present within killed cells or spores. Thus, it can be predicted that as the complexity and thickness of microbial colonies or biofilms increase on spacecraft surfaces, the persistence of ATP will be extended to time periods that are likely to span the duration of the nominal missions. In addition, because biogenic signature molecules appear to persist for many orders of magnitude longer than the viability of individual cells or spores under solar UV irradiation on Mars, spacecraft sanitation and cleanliness protocols may require more stringent controls for biogenic signature molecules than for the presence of viable cells.

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